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(54) METHOD FOR SCREENING DRUGS FOR SUPPRESSING INFLAMMASOME ACTIVITY

VERFAHREN FÜR WIRKSTOFF-SCREENING ZUR UNTERDRÜCKUNG DER INFLAMMASOM-AKTIVITÄT

MÉTHODE DE CRIBLAGE DE MÉDICAMENTS POUR SUPPRIMER L'ACTIVITÉ DE L'INFLAMMASOME

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Description

TECHNICAL FIELD

[0001] The present invention relates to a method for screening drugs for suppressing inflammasome activity.

BACKGROUND ART

[0002] The innate immune response, in which a signal such as amyloid β, asbestos, urate crystals, cholesterol crystals, or oligomers of islet amyloid polypeptides triggers formation of complexes called inflammasomes containing NLRP3, an adaptor protein ASC and caspase-1, followed by activation of caspase-1, leading to production of IL-1β, is considered to be one of the causes of Alzheimer's disease, asbestosis, gout, arteriosclerosis, type 2 diabetes and the like (Halle A, et al., Nat Immunol. 9:857-65,2008; Dostert C. et al., Science, vol. 320,pp. 674-677, 2008; Peter D, et al., Nature 464: 1357-1361, 2010; Mariinon F, et al., Nature 440:237-41, 2006; Masters SL, et al., Nat Immunol. 11:897-904, 2010).

[0003] On the other hand, CINCA (Chronic Infantile Neurologic Cutaneous and Articular) syndrome is one of autoinflammatory syndromes and about a half of patients suffering from this syndrome have a heterozygous mutation in the NLRP3 gene. This mutant NLRP3 gene is said to cause systemic inflammation by constantly activating the above-described inflammasomes.

[0004] *Juliana et al. (2010),* describe that the anti-inflammatory compounds Parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. Furthermore, *Meng et al. (2009)* state that a mutation in the Nlrp3 Gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. In addition, *Meng et al. (2010)* describe new insights into the nature of autoinflammatory diseases from mice with Nlrp3 mutations.

[0005] However, a screening system suitable for developing drugs for suppressing an activity of inflammasome containing NLRP3, which is common among these diseases, has not yet been developed.

DISCLOSURE OF THE INVENTION

[0006] The present invention aims to provide a screening method for development of drugs for suppressing inflammasome activity.

[0007] The inventors of the present invention introduced reprogramming factors into somatic cells of a patient suffering from CINCA syndrome and thereby prepared induced pluripotent stem cells (iPS cells) having mutant NLRP3 gene and iPS cells having wild-type NLRP3 gene from the same patient. These iPS cells were induced to differentiate into macrophages and the resulting macrophages were subjected to LPS stimulation. As a result, macrophages derived from iPS cells having mutant NLRP3 gene were confirmed to produce a much larger amount of IL-1β than macrophages derived from iPS cells having wild-type NLRP3 gene, and it was found that this phenomenon can be used as an index for screening drugs for diseases caused by inflammasomes.

[0008] The inventors of the present invention also carried out differentiation induction into macrophages and the resulting macrophages were subjected to LPS stimulation. As a result, macrophages derived from iPS cells having mutant NLRP3 gene were found to show aggregation of ASC in the cells, and it was found that this phenomenon can be used as an index for screening drugs for diseases caused by inflammasomes.

[0009] The present invention was completed based on these findings.

[0010] It is an aspect of the present invention to provide a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion measured in step (2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0011] It is another aspect of the present invention to provide a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of secretion measured in step (2) is equivalent to or less than the amount of IL-1β secretion from LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene.

[0012] It is another aspect of the present invention to provide a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the respective macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion from the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the amount of IL-1β secretion from the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (2) is equivalent to the amount of IL-1β secretion from macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0013] It is an aspect of the present invention to provide the method as described above, wherein said drug for suppressing inflammasome activity is a therapeutic agent for asbestosis, Alzheimer's disease, type 2 diabetes, atherosclerotic cardiovascular disease, gout, or cryopyrin-associated periodic syndrome.

[0014] It is another aspect of the present invention to provide the method as described above, wherein said mutant NLRP3 gene is NLRP3 gene in which adenine at position 1709 is mutated to guanine.

[0015] It is another aspect of the present invention to provide the method as described above, wherein macrophages derived from iPS cells having wild-type NLRP3 gene are further stimulated with ATP.

[0016] It is another aspect of the present invention to provide the method as described above, wherein said iPS cells having mutant NLRP3 gene and said iPS cells having wild-type NLRP3 gene are iPS cells derived from the same individual.

[0017] It is another aspect of the present invention to provide a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated ASC after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured in step (2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0018] It is another aspect of the present invention to provide a method for screening a drug for suppressing inffammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated ASC after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured in step (2) is equivalent to or less than the ratio of macrophages having aggregated ASC in LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene.

[0019] It is another aspect of the present invention to provide a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated ASC in the respective macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (2) is equivalent to the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0020] It is another aspect of the present invention to provide the method as described above, wherein said drug for suppressing inflammasome activity is a therapeutic agent for asbestosis, Alzheimer's disease, type 2 diabetes, athero-

sclerotic cardiovascular disease, gout, or cryopyrin-associated periodic syndrome.

[0021] It is another aspect of the present invention to provide the method as described above, wherein said mutant NLRP3 gene is NLRP3 gene in which adenine at position 1709 is mutated to guanine.

[0022] It is another aspect of the present invention to provide the method as described above, wherein macrophages derived from iPS cells having wild-type NLRP3 gene are further stimulated with ATP.

[0023] It is another aspect of the present invention to provide the method as described above, wherein said iPS cells having mutant NLRP3 gene and said iPS cells having wild-type NLRP3 gene are iPS cells derived from the same individual.

[0024] Also disclosed is a kit for screening a drug for suppressing inflammasome activity, comprising macrophages derived from iPS cells having mutant NLRP3 gene.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025]

Fig. 1 shows results of sequencing of apart of Exon 3 of NLRP3 gene in (A) wild-type and (B) mutant. It was shown that the nucleotide at position 1709 of NLRP3 is A/G in the mutant.

Fig. 2 shows results of measurement of the amount of IL-1β secretion in iPS cells-derived macrophages having (A) wild-type NLRP3 gene and (B) mutant NLRP3 gene, which measurement was carried out for (I) non-addition group, (2) LPS-addition group and (3) LPS-and ATP-addition group. For the wild type, results from 3 clones WT-1, WT-2 and WT-3, and 201B7 (control) are shown. For the mutant type, results from 3 clones MT-1, MT-2 and MT-3 are shown.

Fig. 3 shows stained images of ASC (left column), stained images by DAPI (middle column) and their superimposed images (right column) in iPS cells-derived macrophages having (A) wild-type NLRP3 gene and (B) mutant NLRP3 gene (photograph). Aggregation of ASC is observed only in cells of the mutant type.

Fig. 4 shows the amount of IL-1β secretion in macrophages derived from iPS cells having mutant NLRP3 gene in the presence of each concentration of cycloheximide (left), SC-514 (middle) and MG132 (right), respectively.

Fig. 5 shows the amount of secretion of IL-1β (A), IL-8 (B) and IL-6 (C), in macrophages derived from iPS cells in the cases of (1) no LPS stimulation and no drug addition, (2) LPS stimulation and no drug addition, (3) LPS stimulation and Interleukin 1 receptor antagonist (IL-1Ra) addition, (4) LPS stimulation and Bay11-7028 (Bay11) addition, (5) LPS stimulation and CA074Me addition, and (6) LPS stimulation and Parthenolide (Parth) addition.

DESCRIPTION OF THE EMBODIMENTS

[0026] The present invention provides a method for screening drugs for suppressing inflammasome activity using macrophages obtained by differentiation induction of induced pluripotent stem cells (iPS cells) having mutant NLRP3 gene and/or iPS cells having wild-type NLRP3 gene. In the present specification, NLRP3 means NLR family, pyrin domain containing 3, which is known to have 5 types of variants, and the NLRP3 may be any of these variants. In the present invention, examples of the NLRP3 include NCBI accession numbers NM_004895 (SEQ ID NO: 1), NM_183395 (SEQ ID NO: 2), NM_001079821 (SEQ ID NO: 3),NM_001127461 (SEQ ID NO: 4) and NM_001127462 (SEQ ID NO: 5). Here, the translation initiation codon in the NLRP3 is preferably the codon located 6 nucleotides downstream of the translation initiation codon described in each of these NCBI accession numbers. Examples of the mutant NLRP3 gene include NLRP3 gene wherein adenine at position 1709 counted from the translation initiation codon (in the case of the coding region shown in the NCBI accession numbers, position 1715 counted from the translation initiation codon) is guanine, cytosine at position 1043 (position 1049 in the coding region shown in the NCBI accession numbers) counted from the translation initiation codon is thymine, or guanine at position 587 (position 593 in the coding region shown in the NCBI accession numbers) counted from the translation initiation codon is adenine. The NLRP3 is preferably the one wherein the nucleotide at position 1079 is mutated to guanine.

Method for Producing iPS Cells

[0027] The iPS cells used in the present invention can be prepared by introducing certain specific nuclear reprogramming substances into somatic cells in the form of DNA or protein, or by increasing expression of endogenous mRNAs and proteins of the nuclear reprogramming substances by an agent(s) (K. Takahashi and S. Yamanaka (2006) Cell, 126:663-676; K. Takahashi et al. (2007) Cell, 131:861-872; J. Yu et al. (2007) Science, 318:1917-1920; M. Nakagawa et al. (2008) Nat. Biotechnol., 26:101-106; WO 2007/069666; and WO 2010/068955). The nuclear reprogramming substances are not restricted as long as these are genes specifically expressed in ES cells, or genes playing important roles in maintenance of the undifferentiated state of ES cells, or gene products thereof, and examples thereof include Oct3/4, Klf4, Klfl, Elf2, Klf5, Sox2, Sox1, Sox3, Sox15, Sox17, Sox18, c-Myc, L-Myc, N-Myc, TERT, SV40 Large T

antigen, HPV16 E6, HPV16 E7, Bmil, Lin28, Lin28b, Nanog, Esrrb and Esrrg. These reprogramming substances may be used in combination when iPS cells are to be established. For example, the combination may contain at least one, two or three of the above reprogramming substances, and the combination preferably contains four of the above reprogramming substances.

[0028] The information on the nucleotide sequences of mouse and human cDNAs of the above-described respective nuclear reprogramming substances, and the amino acid sequences of the proteins encoded by the cDNAs can be obtained by referring to the NCBI accession numbers described in WO 2007/069666. Further, the information on the mouse and human cDNA sequences and amino acid sequences of each of L-Myc, Lin28, Lin28b, Esrrb and Esrrg can be obtained by referring to the NCBI accession numbers described below. Those skilled in the art can prepare desired nuclear reprogramming substances by a conventional method based on the information on the cDNA sequences or amino acid sequences.

Gene name	Mouse	Human
L-Myc	NM_008506	NM_001033081
Lin28	NM_145833	NM_024674
Lin28b	NM_001031772	NM_001004317
Esrrb	NM_011934	NM_004452
Esrrg	NM_011935	NM_001438

[0029] These nuclear reprogramming substances may be introduced into somatic cells in the form of protein by a method such as lipofection, binding to a cell membrane-permeable peptide, or microinjection, or in the form of DNA by a method such as use of a vector including a virus, plasmid and artificial chromosome; lipofection; use of liposomes; or microinjection. Examples of the virus vector include retrovirus vectors, lentivirus vectors (these are described in Cell, 126, pp. 663-676,2006; Cell, 131, pp. 861-872,2007; and Science, 318, pp.1917-1920, 2007), adenovirus vectors (Science, 322, 945-949, 2008), adeno-associated virus vectors and Sendai virus vectors (Proc Jpn Acad Ser B Phys Biol Sci. 85,348-62,2009). Examples of the artificial chromosome vector include human artificial chromosomes (HACs), yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs, PACs). Examples of the plasmid which may be used include plasmids for mammalian cells (Science, 322:949-953, 2008). The vectors may contain a regulatory sequence(s) such as a promoter, enhancer, ribosome binding sequence, terminator and/or polyadenylation site, to allow expression of the nuclear reprogramming substances. Examples of the promoter to be used include the EF1α promoter, CAG promoter, SRα promoter, SV40 promoter, LTR promoter, CMV (cytomegalovinis) promoter, RSV (Rous sarcoma virus) promoter, MoMuLV (Moloney murine leukemia virus) LTR and HSV-TK (herpes simplex virus thymidine kinase) promoter. Among these, the EF1α promoter, CAG promoter, MoMuLV LTR, CMV promoter, SRα promoter and the like are preferred. The vectors may further contain, as required, a sequence of a selection marker such as a drug resistance gene (e.g., kanamycin-resistant gene, ampicillin-resistant gene or puromycin-resistant gene), thymidine kinase gene or diphtheria toxin gene; a gene sequence of a reporter such as the green-fluorescent protein (GFP), β-glucuronidase (GUS) or FLAG; or the like. Further, in order to remove, after introduction of the above vector into somatic cells, the genes encoding the nuclear reprogramming substances, or both the promoters and the genes encoding the reprogramming substances linked thereto, the vector may have loxP sequences in the upstream and the downstream of these sequences. In another preferred mode, a method may be employed wherein, after incorporation of the transgene(s) into a chromosome(s) using a transposon, transposase is allowed to act on the cells using a plasmid vector or an adenovirus vector, thereby completely removing the transgene(s) from the chromosome(s). Preferred examples of the transposon include piggyBac, which is a transposon derived from a lepidopteran insect (Kaji, K. et al., (2009), Nature, 458: 771-775; Woltjen et al., (2009), Nature, 458: 766-770; and WO 2010/012077). Further, the vector may contain the replication origin of lymphotrophic herpes virus, BK virus or Bovine papillomavirus and sequences involved in their replication, such that the vector can replicate without being incorporated into the chromosome and exist episomally. Examples of such a vector include vectors containing EBNA-1 and oriP sequences and vectors containing Large T and SV40ori sequences (WO 2009/115295; WO 2009/157201; WO 2009/149233). Further, in order to introduce plural nuclear reprogramming substances at the same time, an expression vector which allows polycistronic expression may be used. In order to allow polycistronic expression, the sequences encoding the genes may be linked to each other via IRES or the foot-and-mouth disease virus (FMDV) 2A coding region (Science, 322:949-953,2008; WO 2009/092042 and WO 2009/152529).

[0030] For enhancing the induction efficiency of iPS cells upon the nuclear reprogramming, histone deacetylase (HDAC) inhibitors [for example, low molecular inhibitors such as valproic acid (VPA) (Nat. Biotechnol., 26(7): 795-797 (2008)), trichostatin A, sodium butyrate, MC 1293 and M344; and nucleic acid-type expression inhibitors such as siRNAs and shRNAs against HDAC (e.g., HDAC1 siRNA Smartpool (registered trademark) (Millipore) and HuSH 29mer shRNA Constructs against HDAC1 (OriGene))], DNA methyltransferase inhibitors (e.g., 5'-azacytidine) (Nat. Biotechnol., 26(7): 795-797 (2008)), G9a histone methyltransferase inhibitors [for example, low molecular inhibitors such as BIX-01294

(Cell Stem Cell, 2: 525-528 (2008)); and nucleic acid-type expression inhibitors such as siRNAs and shRNAs against G9a (e.g., G9a siRNA (human) (Santa Cruz Biotechnology))], L-channel calcium agonists (e.g., Bayk8644) (Cell Stem Cell, 3, 568-574 (2008)), p53 inhibitors [e.g., siRNAs and shRNAs against p53 (Cell Stem Cell, 3,475-479 (2008))], Wnt Signaling activators (e.g., soluble Wnt3a) (Cell Stem Cell, 3, 132-135 (2008)), growth factors such as LIF and bFGF, ALK5 inhibitors (e.g., SB431542) (Nat Methods, 6:805-8 (2009)), mitogen-activated protein kinase signaling inhibitors, glycogen synthase kinase-3 inhibitors (PLoS Biology, 6(10), 2237-2247 (2008)), miRNAs such as miR-291-3p, miR-294 and miR-295 (R.L. Judson et al., Nat. Biotech., 27:459-461 (2009)), and the like may be used in addition to the above-described factors.

[0031] Examples of the agent(s) used in the method for increasing expression of the endogenous proteins of nuclear reprogramming substances using an agent include 6-bromoindirubin-3'-oxime, indirubin-5-nitre-3'-oxime, valproic acid, 2-(3-(6-methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine,1-(4-methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2H)-benzothiazolyl)ethanone HBr(pifithrin-alpha), prostaglandin J2 and prostaglandin E2 (WO 2010/068955).

[0032] Examples of the culture medium for induction of the iPS cells include (1) DMEM, DMEM/F12 and DME media supplemented with 10 to 15% FBS (these media may further contain LIF, penicillin/streptomycin, puromycin, L-glutamine, non-essential amino acids, β-mercaptoethanol and/or the like, as appropriate); (2) culture media for ES cells containing bFGF or SCF, for example, culture media for mouse ES cells (e.g., TX-WES medium, Thromb-X) and culture media for primate ES cells (e.g., culture medium for primate (human and monkey) ES cells (ReproCELL Inc., Kyoto, Japan), mTeSR-1).

[0033] Examples of the culture method include a method wherein somatic cells and nuclear reprogramming substances (DNAs or proteins) are brought into contact with each other at 37°C in the presence of 5% CO_2 in DMEM or DMEM/F12 medium supplemented with 10% FBS, and the cells are cultured for about 4 to 7 days, followed by replating the cells on feeder cells (e.g., mitomycin C-treated STO cells or SNL cells) and starting culture in a bFGF-containing culture medium for primate ES cells about 10 days after the contact between the somatic cells and the reprogramming substances, thereby allowing ES cell-like colonies to appear about 30 to about 45 days after the contact, or later. To enhance the induction efficiency of iPS cells, the culture may be carried out under a condition wherein the concentration of oxygen is as low as 5 to 10%.

[0034] As an alternative culture method, the somatic cells may be cultured on feeder cells (e.g., mitomycin C-treated STO cells or SNL cells) in DMEM medium supplemented with 10% FBS (which may further contain LIF, penicillin/streptomycin, puromycin, L-glutamine, non-essential amino acids, β-mercaptoethanol and/or the like, as appropriate), thereby allowing ES-like colonies to appear after about 25 to about 30 days of the culture, or later.

[0035] During the above culture, the culture medium is replaced with a fresh culture medium once every day from Day 2 of the culture. The number of the somatic cells used for nuclear reprogramming is not restricted, and usually within the range of about 5×10^3 to about 5×10^6 cells per 100-cm^2 area on the culture dish.

[0036] In cases where a gene including a drug resistance gene is used as a marker gene, cells expressing the marker gene can be selected by culturing the cells in a culture medium (selection medium) containing the corresponding drug. Cells expressing a marker gene can be detected by observation under a fluorescence microscope in cases where the marker gene is the gene of a fluorescent protein; by adding a luminescent substrate in cases where the marker gene is the gene of luciferase; or by adding a coloring substrate in cases where the marker gene is the gene of a coloring enzyme.

[0037] Examples of the "somatic cells" used in the present specification include epithelial cells which are keratinized (e.g., keratinized epidermal cells), mucosal epithelial cells (e.g., epithelial cells of the lingual surface), epithelial cells of exocrine glands (e.g., mammary cells), hormone-secreting cells (e.g., adrenomedullary cells), cells for metabolism and storage (e.g., hepatic cells), luminal epithelial cells constituting boundary surfaces (e.g., type I alveolar cells), luminal epithelial cells in the closed circulatory system (e.g., vascular endothelial cells), ciliated cells having a carrying capacity (e.g., tracheal epithelial cells), extracellular matrix-secreting cells (e.g., fibroblasts), contractile cells (e.g., smooth muscle cells), cells involved in the blood system and the immune system (e.g., T lymphocytes), sensory cells (e.g., rod cells), autonomic neurons (e.g., cholinergic neurons), supporting cells of sense organs and peripheral neurons (e.g., satellite cells), nerve cells and glial cells in the central nervous system (e.g., astroglial cells) and pigment cells (e.g., retinal pigment epithelial cells), and progenitor cells (tissue progenitor cells) thereof The level of differentiation of the somatic cells is not restricted, and either undifferentiated progenitor cells (including somatic stem cells) or terminally-differentiated mature cells may be similarly used as the source of the somatic cells in the present invention. Here, examples of the undifferentiated progenitor cells include tissue stem cells (somatic stem cells) such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells and dental pulp stem cells.

[0038] In the present invention, somatic cells having a mutation of NLRP3 and somatic cells having no such a mutation of NLRP3 are preferably obtained from the same individual. The iPS cells are preferably established from a patient suffering from CINCA (Chronic Infantile Neurologic Cutaneous and Articular).

[0039] The mutant NLRP3 gene may be one which has been originally retained by the somatic cells from which the iPS cells are derived, or the mutation may be introduced to NLRP3 gene using homologous recombination after establishment of the iPS cells. The homologous recombination may be carried out using a method well known to those skilled

in the art.

Method of Differentiation Induction into Macrophages

[0040] For producing macrophages from the thus obtained iPS cells, a differentiation induction method comprising the following steps may be used:

(1) culturing iPS cells on OP9 cells;
(2) separating cells using an angioblast marker as an index;
(3) culturing the separated cells on OP9 cells; and
(4) separating/purifying cells using a macrophage marker as an index.

[0041] The macrophages in the present invention are cells expressing any or all of CD11b, CD 14 and CD68, preferably cells expressing CD 14. Examples of the angioblast marker include CD34, KDR and TRA-1-85.

[0042] Prior to Step (1), the iPS cells may be dissociated by an arbitrary method. The dissociation may be carried out either mechanically or by using a dissociation solution having a protease activity and a collagenase activity (e.g., Accutase(TM), Accumax(TM) or a cell detachment liquid for primate ES cells (ReproCELL Inc.)) or a separation liquid having only a collagenase activity.

[0043] Examples of the coating agent employed in Step (1) and Step (3) include Matrigel (BD), type I collagen, type IV collagen, gelatin, laminin, heparan sulfate proteoglycan and entactin, and combinations thereof.

[0044] The culture medium for producing macrophages can be prepared by using, as a basal medium, a culture medium used for culturing animal cells. Examples of the basal medium include IMDM medium, Medium 199 medium, Eagle's Minimum Essential Medium (EMEM), αMEM medium, Dulbecco's modified Eagle's Medium (DMEM), Ham's F12 medium, RPMI 1640 medium and Fischer's medium, and mixed media thereof The culture medium is preferably αMEM medium. Further, the culture medium may be either a serum-containing medium or a serum-free medium. As required, the culture medium may contain one or more of serum replacements such as albumin, transferrin, Knockout Serum Replacement (KSR) (a serum replacement of FBS for culturing ES cells), fatty acid, insulin, collagen precursor, minor element, 2-mercaptoethanol and 3'-thiolglycerol; and/or may contain one or more of lipids, amino acids, L-glutamine, Glutamax (Invitrogen), non-essential amino acids, vitamins, antibiotics, antioxidants, pyruvic acid, buffers, inorganic salts, N2 supplement (Invitrogen), B27 supplement (Invitrogen), cytokines such as VEGF, stem cell factor (SCF), IL-3, thrombopoietin (TPO), FLT-3 ligand (FL) and macrophage colony-stimulating factor (M-CSF), and the like. Examples of a preferred medium include αMEM containing 10% FCS and VEGF in Step (1), and αMEM containing 10% FCS, SCF, IL-3, TPO, FL and M-CSF in Step (3).

[0045] The culture temperature is not restricted and may be about 30 to 40°C, preferably about 37°C, and the culture is carried out under atmosphere of CO_2-containing air, wherein the CO_2 concentration is preferably about 2 to 5%. The culturing time is not restricted, and, for example, 5 days to 15 days, more preferably 10 days in Step (1); and, for example, 10 days to 20 days, more preferably 16 days in Step (3).

[0046] Angioblast marker-positive cells or macrophage marker-positive cells can be separated/purified from cells stained with an antibody for each marker using a flow cytometer or magnetic beads having the antibody by a method well known to those skilled in the art.

Screening Method

[0047] The present invention provides a method for screening a drug for suppressing inflammasome activity. The inflammasomes means complexes composed of NLRP3, ASC and caspase-1. The activation of inflammasomes is not restricted, and, for example, it means activation of caspase-1 via formation of the above complexes.

[0048] The drug for suppressing inflammasome activity can be used as a therapeutic agent or prophylactic agent for diseases caused by activation of inflammasomes, and examples of the diseases caused by activation of inffammasomes include, but are not limited to, asbestosis, Alzheimer's disease, type 2 diabetes, atherosclerotic cardiovascular disease, gout and cryopyrin-associated periodic syndrome.

[0049] Inflammasome activity does not increase upon LPS stimulation in macrophages derived from iPS cells having wild-type NLRP3 gene whereas inflammasome activity increases upon LPS stimulation in macrophages derived from iPS cells having mutant NLRP3 gene. Thus, in cases where macrophages derived from iPS cells having mutant NLRP3 gene obtained by the above-mentioned method are used, drugs for suppressing inflammasome activity can be screened by:

(1-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;

(1-2) measuring the amount of IL-1β secretion from the macrophages after step (1-1); and
(1-3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion measured in step (1-2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0050] Here, the LPS stimulation is carried out by adding LPS to the culture of the macrophages. In addition to the LPS stimulation, stimulation with ATP may be carried out. The stimulation time is not restricted and may be, for example, 2 hours to 24 hours in the case of LPS, and 10 minutes to 1 hour in the case of ATP.

[0051] In the present invention, the amount of secretion of IL-1β can be measured using the culture supernatant of the macrophages, and examples of the measurement method include immunoassays. Examples of the immunoassays include the radioimmunoassay, enzyme immunoassay, fluoroimmunoassay, luminescence immunoassay, immunoprecipitation, turbidimetric immunoassay, Western blotting and immunodiffusion, and the immunoassay is preferably the enzyme immunoassay, especially preferably the enzyme-linked immunosorbent assay (ELISA) (e.g., sandwich ELISA).

[0052] Another example of the method for screening drugs for suppressing inflammasome activity includes a method comprising the steps of:

(2-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2-2) measuring the ratio of macrophages having aggregated ASC after step (2-1); and
(2-3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured in step (2-2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0053] In the present invention, ASC is the apoptosis-associated speck-like protein containing a CARD, which is a protein also referred to as PYCARD. Examples of variants of the gene encoding ASC include, but are not limited to, NCBI accession numbers NM_013258 and NM_145182.

[0054] Aggregation of ASC means that ASC is not widely distributed in the cell and is localized to form clusters. Aggregation of ASC occurs when macrophages are stimulated with LPS, and can be preferably judged by stronger staining of the areas of localization than the other areas when cells are immunostained using an anti-ASC antibody. An example of preferred aggregation is shown in the left column in Fig. 3B.

[0055] The ratio of macrophages having aggregated ASC may be the number of macrophages having aggregated ASC per total macrophages or a certain number of macrophages (e.g., per 10,000 macrophages) or the number of macrophages having aggregated ASC in a culture dish. The aggregation of ASC may be either a single aggregate or a plurality of aggregates in each macrophage.

[0056] Other examples of the method for screening drugs for suppressing inflammasome, activity include a method comprising the steps of:

(3-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(3-2) measuring the amount of IL-1β secretion from the macrophages after step (3-1); and
(3-3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of secretion measured in step (3-2) is equivalent to or less than the amount of IL-1β secretion from LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;

and a method comprising the steps of:

(4-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(4-2) measuring the ratio of macrophages having aggregated ASC after step (4-1); and
(4-3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured in step (4-2) is equivalent to or less than the ratio of macrophages having aggregated ASC in LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene.

[0057] The term "equivalent" includes a case where the value is strictly identical as well as a case where an error preferably within the range of approximately ±5% or more preferably within the range of approximately ±1% exists with respect to the measured value.

[0058] In the present invention, the LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene may be further stimulated with ATP before measuring the amount of 1L-1β secretion.

[0059] Other examples of the method for screening drugs for suppressing inflammasome activity include a method comprising the steps of:

(5-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(5-2) measuring the amount of IL-1β secretion from the respective macrophages after step (5-1); and
(5-3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion from the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (5-2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the amount of IL-1β secretion from the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (5-2) is equivalent to the amount of IL-1β secretion from macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance; and a method comprising the steps of:
(6-1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(6-2) measuring the ratio of macrophages having aggregated ASC in the respective macrophages after step (6-1); and
(6-3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (6-2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (6-2) is equivalent to the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

[0060] In the screening method of the present invention, an arbitrary test substance can be used, and examples of the test substance include cell extracts, cell culture supernatants, microbial fermentation products, extracts derived from marine organisms, plant extracts, purified proteins and crude proteins, peptides, nonpeptide compounds, synthetic low molecular compounds and naturally occurring compounds. The test compounds can be obtained by using any of a number of approaches in combinatorial library methods known in the art, such as (1) the biological library method, (2) the synthetic library method using deconvolution, (3) the "one-bead one-compound" library method and (4) the synthetic library method using affinity chromatography selection. Application of the biological library method using affinity chromatography selection is limited to peptide libraries, but the other types of approaches can be applied to low-molecular compound libraries of peptides, nonpeptide oligomers or compounds (Lam (1997) Anticancer Drug Des. 12:145-67). Examples of synthetic methods of molecular libraries are shown in the art (DeWitt et al. (1993) Proc. Natl. Acad. Sci. USA 90: 6909-13; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91: 11422-6; Zuckermann et al. (1994) J. Med. Chem. 37: 2678-85; Cho et al. (1993) Science 261: 1303-5; Carell et al. (1994) Angew. Chem. Int. Ed. Engl. 33: 2059; Carell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2061; Gallop et al. (1994) J. Med. Chem. 37: 1233-51). The compound libraries may be prepared as solutions (see Houghten (1992) BiolTechniques 13: 412-21) or beads (Lam (1991) Nature 354: 82-4), chips (Fodor (1993) Nature 364: 55-6), bacteria (US 5,223,409 B), spores (US 5,571,698 B, US 5,403,484 B and US 5,223,409 B), plasmids (Cull et al. (1992) Proc. Natl. Acad. Sci. USA 89: 1865-9) or phages (Scott and Smith (1990) Science 249:386-90; Devlin (1990) Science 249: 404-6; Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA 87: 6378-82; Felici (1991) J. Mol. Biol. 222:301-10; US 2002-103360).

Kit for Screening Drugs

[0061] Also described is a kit for screening drugs for suppressing inflammasome activity. This kit may comprise the above-mentioned cells, reagents and culture medium. The kit may further comprise a document or an instruction that describes a protocol for differentiation induction.

EXAMPLES

Establishment of iPS Cells

[0062] Using a retrovirus that expresses human OCT3/4, human SOX2, human KLF4 and human c-MYC as described in Takahashi, K. et al., Cell, 131: 861-872 (2007), 6 clones of iPS cells were established from fibroblasts established from the skin obtained by biopsy from a patient suffering from Chronic Infantile Neurologic, Cutaneous, Articular syndrome (CINCA syndrome) with the patient's consent. Since the patient was a mosaic CINCA syndrome patient, these established iPS cells, irrespective of the fact that these were obtained from the same individual, contained 3 clones having wild-type

NLRP3 gene and the other clones having mutant NLRP3 gene in which alanine at position 1709 was mutated to guanine in one of the alleles (Fig. 1).

Method of Differentiation Induction into Macrophages

[0063] The iPS cells obtained by the above method were dispersed using CTK solution (Suemori, H et al., Biochem Biophys Res Commun, 35: 926-932 (2006)) and left to stand in a gelatin-coated dish at 37°C for 60 minutes to remove feeder cells, followed by plating the cells on OP9 cells (Nishikawa, S.I. et al, Development 125,1747-1757 (1998)). The culture medium used at this time was αMEM containing 10% FCS, antibiotics-antimycotic and 10 ng/ml VEGF. Ten days after the start of the culture, the cells were dispersed using trypsin, and Tra-1-85-positive/CD34-positive/KDR-positive cells were separated and collected using FACS, followed by plating the collected cells on OP9 cells. The culture medium used at this time was αMEM containing 10% FCS, antibiotics-antimycotic, 50 ng/ml hSCF, 50 ng/ml IL-3,10 ng/ml TPO, 50 ng/ml FL and 50 ng/ml M-CSF. Subsequently, 16 days after the separation and collection of the cells, the cells were suspended using Accumax, and CD14-positive cells were purified by Magnetic activated cell sorting. The CD 14-positive cells obtained from the 6 clones of iPS cells were used as the macrophages derived from iPS cells, and the 3 macrophages having wild-type NLRP3 gene were designated WT-1, WT-2 and WT-3, respectively, and the 3 macrophages having mutant NLRP3 gene were designated MT-1, MT-2 and MT-3, respectively. Further, control macrophages (201B7) were obtained from 201B7 described in Takahashi, K. et al., Cell, 131: 861-872 (2007) by the same method.

Measurement of Amount of Production of IL-1β

[0064] The iPS cells-derived macrophages obtained by the above method were suspended in RPMI medium supplemented with 10% FCS and divided into 3 groups, that is, (1) non-addition group, (2) a group in which LPS (Lipopolysaccharide) was added to the medium at 1 μg/ml and the macrophages were then cultured for 4 hours, and (3) a group in which LPS (Lipopolysaccharide) was added to the medium at 1 μg/ml and the macrophages were then cultured for 4 hours, followed by adding ATP (Adenosine triphosphate) to the medium at 1 mM and then culturing the macrophages for 30 minutes. Thereafter, the culture supernatant in each group was recovered. The recovered culture supernatant was subjected to flow cytometry to measure the concentration of IL-1β using Human Th1/Th2 11plex Kit (BMS) according to the attached protocol (Fig. 2). The measurement was carried out 3 times in the same manner for each group. As a result, it was observed that a larger amount of IL-1β was secreted by LPS stimulation, from the macrophages having the mutant NLRP3 gene than from the macrophages having wild-type NLRP3 gene. The stimulation with ATP did not cause any change.

Intracellular Distribution of ASC

[0065] LPS (Lipopolysaccharide) was added to a culture medium at 1 μg/ml and macrophages derived from iPS cells having mutant NLRP3 gene were cultured therein for 24 hours, followed by adding ATP (Adenosine triphosphate) to the medium at 1 mM and then culturing the macrophages for 30 minutes. Immunostaining was carried out to measure the intracellular distribution of ASC (Apoptotic speck-like protein containing a CARD) using an anti-ASC antibody. As a result, a large number of cells presenting aggregation of ASC, which is an index of activation of inflammasomes composed of NLRP3, ASC and caspase-1, were observed in the LPS/ATP-stimulated group (Fig. 3B). On the other hand, in macrophages derived from iPS cells having wild-type NLRP3 gene, which macrophages were not stimulated with LPS and ATP, ASC was distributed throughout the cell and aggregation of ASC was not observed (Fig. 3A). From the above results, it was found that, in macrophages derived from iPS cells having mutant NLRP3 gene, inflammasomes are activated by stimulation with LPS and ATP.

Evaluation of Drug Effects

[0066] The macrophage MT-1 induced from iPS cells as described above was added with each concentration of cycloheximide (CHX) (Sigma), SC-514 (Tocris) or MG132 (Calbiochem) and cultured for two hours, and the medium was replaced with a medium containing each drug and LPS (1μg/ml), and culture was performed for another four hours, followed by measurement of the amount of IL-1β secretion using Human Th1/Th2 11plex Kit (Fig. 4). It was confirmed that NF-κB inhibitor SC-514 and MG132 inhibited IL-1β secretion in a dose dependent manner, as in the case of CHX used as a control.

[0067] The macrophage MT-1 induced from iPS cells as described above was added with 100ng/ml of interleukin 1 receptor antagonist (IL-1Ra) (R&D Systems), 12μM of Bay11-7028 (Sigma), 50μM of CA074Me (Calbiochem) or 10μM of Parthenolide (Parth) and cultured for two hours, and the medium was replaced with a medium containing each drug and LPS (1μg/ml), and culture was performed for another four hours, followed by measurement of the amount of secretion

of IL-1β (Fig. 5A), IL-8 (Fig. 5B), IL-6 (Fig. 5C) using Human Th1/Th2 11plex Kit. It was found that the secretion amount of the three types of interleukins did not change with the addition of IL-1Ra whereas the secretion amount of the three types of interleukins was inhibited with Bay11-7028 and Parth. On the other hand, CA074Me specifically inhibited the secretion amount of IL-1β. These results show that drug effects can be evaluated by using macrophages induced from iPS cells derived from the CINCA syndrome patient

INDUSTRIAL APPLICABILITY

[0068] The method of the present invention is useful for screening a drug such as a therapeutic agent for asbestosis, Alzheimer's disease, type 2 diabetes, atherosclerotic cardiovascular disease, gout, or cryopyrin-associated periodic syndrome.

[0069] In this specification, the test substance can be provided as the drug candidate compound.

[0070] While the present invention has been described with emphasis on preferred embodiments, it is obvious to those skilled in the art that the preferred embodiments can be modified. For example, the motivation to generate a certain drug through the combination or the synthesis of the screened drug candidate compound with other chemical compound is not precluded under the present invention. The present invention intends that the present invention can be embodied by methods other than those described in detail in the present specification. Accordingly, the present invention encompasses all modifications encompassed in the appended "CLAIMS."

SEQUENCE LISTING

[0071]

<110> Kyoto University

<120> METHOD FOR SCREENING DRUGS FOR SUPPRESSING INFLAMMASOME ACTIVITY

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<150> US 61/415102
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Claims

1. A method selected from any one of (A), (B) and (C):

(A) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with lipopolysaccharide (LPS)-stimulated macrophages derived from induced pluripotent stem cells (iPS cells) having mutant NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion measured in step (2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance;

(B) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of secretion measured in step (2) is equivalent to or less than the amount of IL-1β secretion from LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;

(C) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(2) measuring the amount of IL-1β secretion from the respective macrophages after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the amount of IL-1β secretion from the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (2) is smaller than the amount of IL-1β secretion from macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the amount of IL-1β secretion from the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (2) is equivalent to the amount of IL-1β secretion from macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

2. The method according to claim 1 , wherein said drug for suppressing inflammasome activity is a therapeutic agent for asbestosis, Alzheimer's disease, type 2 diabetes, atherosclerotic cardiovascular disease, gout, or cryopyrin-associated periodic syndrome.

3. The method according to claim 1 or 2, wherein said mutant NLRP3 gene is NLRP3 gene in which adenine at position 1709 is mutated to guanine.

4. The method according to claim 1 (B) or 1 (C), wherein macrophages derived from iPS cells having wild-type NLRP3 gene are further stimulated with ATP.

5. The method according to claim 1 (B) or 1 (C), wherein said iPS cells having mutant NLRP3 gene and said iPS cells having wild-type NLRP3 gene are iPS cells derived from the same individual.

6. A method selected from any one of (A), (B) and (C):

(A) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated Apoptotic speck-like protein containing a CARD (ASC) after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured

in step (2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance;

(B) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated ASC after step (1); and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio measured in step (2) is equivalent to or less than the ratio of macrophages having aggregated ASC in LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;

(C) a method for screening a drug for suppressing inflammasome activity, comprising the steps of:

(1) contacting a test substance with LPS-stimulated macrophages derived from iPS cells having mutant NLRP3 gene and with LPS-stimulated macrophages derived from iPS cells having wild-type NLRP3 gene;
(2) measuring the ratio of macrophages having aggregated ASC in the respective macrophages after step (1) ; and
(3) selecting the test substance as a drug for suppressing inflammasome activity when the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having mutant NLRP3 gene measured in step (2) is smaller than the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having mutant NLRP3 gene which are stimulated with LPS but not contacted with the test substance, and the ratio of macrophages having aggregated ASC in the macrophages derived from iPS cells having wild-type NLRP3 gene measured in step (2) is equivalent to the ratio of macrophages having aggregated ASC in macrophages derived from iPS cells having wild-type NLRP3 gene which are stimulated with LPS but not contacted with the test substance.

7. The method according to claim 6, wherein said drug for suppressing inflammasome activity is a therapeutic agent for asbestosis, Alzheimer's disease, type 2 diabetes, atherosclerotic cardiovascular disease, gout, or cryopyrin-associated periodic syndrome.

8. The method according to claim 6, wherein said mutant NLRP3 gene is NLRP3 gene in which adenine at position 1709 is mutated to guanine.

9. The method according to claim 6 (B) or 6 (C), wherein macrophages derived from iPS cells having wild-type NLRP3 gene are further stimulated with ATP.

10. The method according to claim 6 (B) or 6 (C), wherein said iPS cells having mutant NLRP3 gene and said iPS cells having wild-type NLRP3 gene are iPS cells derived from the same individual.

Patentansprüche

1. Verfahren, ausgewählt aus irgendeinem Verfahren nach (A), (B) und (C):

(A) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit Lipopolysaccharid (LPS)-stimulierten Makrophagen, die aus induzierten, pluripotenten Stammzellen (iPS-Zellen) mit mutiertem NLRP3-Gen stammen;
(2) Messen der Menge der IL-1β-Sekretion aus den Makrophagen nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls die in Schritt (2) gemessene Menge der IL-1β-Sekretion geringer als die Menge der IL-1β-Sekretion aus Makrophagen ist, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden;

(B) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen;
(2) Messen der Menge der IL-1β-Sekretion aus den Makrophagen nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls die in Schritt (2) gemessene Menge der Sekretion der Menge der IL-1β-Sekretion aus LPS-stimulierten Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, entspricht oder geringer ist;

(C) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, und mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen;
(2) Messen der Menge der IL-1β-Sekretion aus den entsprechenden Makrophagen nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls die in Schritt (2) gemessene Menge der IL-1β-Sekretion aus den Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, geringer als die Menge der IL-1β-Sekretion aus Makrophagen ist, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden, und die in Schritt (2) gemessene Menge der IL-1β-Sekretion aus den Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, der Menge der IL-1β-Sekretion aus Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden, entspricht.

2. Verfahren gemäß Anspruch 1, wobei dieses Medikament zur Unterdrückung der Inflammasom-Aktivität ein Therapeutikum für Asbestose, Morbus Alzheimer, Typ 2-Diabetes, arteriosklerotische kardiovaskuläre Erkrankung, Gicht oder Cryopyrin-assoziiertes periodisches Syndrom ist.

3. Verfahren gemäß Anspruch 1 oder 2, wobei dieses mutierte NLRP3-Gen ein NLRP3-Gen ist, in dem Adenin an Position 1709 zu Guanin mutiert ist.

4. Verfahren gemäß Anspruch 1 (B) oder 1 (C), wobei die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammenden Makrophagen ferner mit ATP stimuliert sind.

5. Verfahren gemäß Anspruch 1 (B) oder 1 (C), wobei diese iPS-Zellen mit mutiertem NLRP3-Gen und diese iPS-Zellen mit Wildtyp-NLRP3-Gen iPS-Zellen sind, die aus dem gleichen Individuum stammen.

6. Verfahren, ausgewählt aus einem Verfahren nach (A), (B) und (C):

(A) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen;
(2) Messen des Anteils von Makrophagen mit aggregiertem, apoptotischem Speck-like Protein, enthaltend ein CARD (ASC), nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls der in Schritt (2) gemessene Anteil geringer ist als der Anteil von Makrophagen mit aggregiertem ASC in Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden;

(B) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen;
(2) Messen des Anteils von Makrophagen mit aggregiertem ASC nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls der in Schritt (2) gemessene Anteil dem Anteil von Makrophagen mit aggregiertem ASC in LPS-stimulierten

Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, entspricht oder geringer ist;

(C) Verfahren zum Screening eines Medikaments zur Unterdrückung von Inflammasom-Aktivität, umfassend die folgenden Schritte:

(1) In Kontakt bringen einer Testsubstanz mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, und mit LPS-stimulierten Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen;
(2) Messen des Anteils von Makrophagen mit aggregiertem ASC in den entsprechenden Makrophagen nach Schritt (1); und
(3) Auswählen der Testsubstanz als ein Medikament zur Unterdrückung von Inflammasom-Aktivität, falls der in Schritt (2) gemessene Anteil von Makrophagen mit aggregiertem ASC in den Makrophagen, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, geringer als der Anteil von Makrophagen mit aggregiertem ASC in Makrophagen ist, die aus iPS-Zellen mit mutiertem NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden, und der in Schritt (2) gemessene Anteil von Makrophagen mit aggregiertem ASC in den Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, dem Anteil von Makrophagen mit aggregiertem ASC in den Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, die mit LPS stimuliert werden, aber nicht mit der Testsubstanz in Kontakt gebracht werden, entspricht.

7. Verfahren gemäß Anspruch 6, wobei dieses Medikament zur Unterdrückung von Inflammasom-Aktivität ein Therapeutikum für Asbestose, Morbus Alzheimer, Typ 2-Diabetes, arteriosklerotische kardiovaskuläre Erkrankung, Gicht oder Cryopyrin-assoziiertes periodisches Syndrom ist.

8. Verfahren gemäß Anspruch 6, wobei dieses mutierte NLRP3-Gen ein NLRP3-Gen ist, in dem Adenin an Position 1709 zu Guanin mutiert ist.

9. Verfahren gemäß Anspruch 6 (B) oder 6 (C), wobei die Makrophagen, die aus iPS-Zellen mit Wildtyp-NLRP3-Gen stammen, ferner mit ATP stimuliert sind.

10. Verfahren gemäß Anspruch 6 (B) oder 6 (C), wobei diese iPS-Zellen mit mutiertem NLRP3-Gen und diese iPS-Zellen mit Wildtyp-NLRP3-Gen iPS-Zellen sind, die aus dem gleichen Individuum stammen.

Revendications

1. Procédé choisi parmi l'un quelconque de (A), (B) et (C) :

(A) Procédé de criblage un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés par un lipopolysaccharide (LPS), dérivés de cellules souches pluripotentes induites (cellules iPS) ayant un gène mutant NLRP3 ;
(2) mesurer la quantité de sécrétion d'IL-1β des macrophages après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la quantité de sécrétion d'IL-1β mesurée à l'étape (2) est plus petite que la quantité de sécrétion d'IL-1β des macrophages, stimulés avec du LPS, dérivés des cellules iPS ayant un gène mutant NLRP3, mais sans venir en contact avec la substance test ;

(B) Procédé de criblage d'un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés par un LPS, dérivés de cellules iPS ayant un gène mutant NLRP3 ;
(2) mesurer la quantité de sécrétion d'IL-1β des macrophages après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la quantité de sécrétion mesurée à l'étape (2) est équivalente ou inférieure à la quantité de sécrétion d'IL-1β des macrophages, stimulés par un LPS, dérivés des cellules iPS ayant un gène de type sauvage NLRP3 ;

(C) Procédé de criblage d'un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés par un LPS, dérivés de cellules iPS ayant un gène mutant NLRP3 et avec des macrophages, stimulés par un LPS, dérivés des cellules iPS ayant un gène de type sauvage NLRP3 ;
(2) mesurer la quantité de sécrétion d'IL-1β des macrophages respectifs après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la quantité de sécrétion d'IL-1β des macrophages dérivés des cellules iPS ayant un gène mutant NLRP3 mesurée à l'étape (2) est plus petite que la quantité de sécrétion d'IL-1β des macrophages dérivés des cellules iPS ayant un gène mutant NLRP3 qui sont stimulés par un LPS, mais sans venir en contact avec la substance test, et la quantité de sécrétion d'IL-1β des macrophages dérivés des cellules iPS ayant un gène de type sauvage NLRP3 mesurée à l'étape (2) est équivalente à la quantité de sécrétion d'IL-1β des macrophages dérivés des cellules iPS ayant un gène de type sauvage NLRP3 qui sont stimulés par un LPS, mais sans venir en contact avec la substance test.

2. Procédé selon la revendication 1, dans lequel ledit médicament pour supprimer l'activité d'un inflammasome est un agent thérapeutique pour l'amiantose, la maladie d'Alzheimer, un diabète de type 2, les maladies cardiovasculaires athéroscléreuses, la goutte ou un syndrome périodique associé à la cryopyrine.

3. Procédé selon la revendication 1 ou 2, dans lequel ledit gène mutant NLRP3 est un gène NLRP3 dans lequel l'adénine en position 1709 est mutée en guanine.

4. Procédé selon la revendication 1 (B) ou 1 (C), dans lequel des macrophages dérivés des cellules iPS ayant un gène de type sauvage NLRP3 sont en outre stimulés par de l'ATP.

5. Procédé selon la revendication 1 (B) ou 1 (C), dans lequel lesdites cellules iPS ayant un gène mutant NLRP3 et lesdites cellules iPS ayant un gène de type sauvage NLRP3 sont des cellules iPS dérivées de la même personne.

6. Procédé choisi parmi l'un quelconque de (A), (B) et (C) :

(A) Procédé de criblage d'un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés avec du LPS, dérivés de cellules iPS ayant un gène mutant NLRP3 ;
(2) mesurer la teneur en macrophages ayant une protéine mouchetée apoptotique agrégée contenant un CARD (ASC) après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la teneur mesurée à l'étape (2) est plus faible que la teneur des macrophages ayant de l'ASC agrégé dans des macrophages dérivés de cellules iPS ayant un gène mutant NLRP3 qui sont stimulés par un LPS, mais sans venir en contact avec la substance test ;

(B) Procédé de criblage d'un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés par un LPS, dérivés de cellules iPS ayant un gène mutant NLRP3 ;
(2) mesurer la teneur en macrophages ayant de l'ASC agrégé après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la teneur mesurée à l'étape (2) est équivalente ou inférieure à la teneur en macrophages ayant de l'ASC agrégé dans des macrophages, stimulés par un LPS, dérivés des cellules iPS ayant un gène de type sauvage NLRP3 ;

(C) Procédé de criblage d'un médicament pour supprimer l'activité d'un inflammasome, comprenant les étapes consistant à :

(1) mettre en contact une substance test avec des macrophages, stimulés par un LPS, dérivés de cellules

iPS ayant un gène mutant NLRP3 et avec des macrophages, stimulés par un LPS, dérivés des cellules iPS ayant un gène de type sauvage NLRP3 ;
(2) mesurer la teneur en macrophages ayant de l'ASC agrégé après l'étape (1) ; et
(3) choisir la substance test en tant que médicament pour supprimer l'activité de l'inflammasome lorsque la teneur en macrophages ayant un ASC agrégé dans les macrophages dérivés de cellules iPS ayant un gène mutant NLRP3 mesurée l'étape (2) est plus faible que la teneur en macrophages ayant un ASC agrégé dans les macrophages dérivés des cellules iPS ayant un gène mutant NLRP3 qui sont stimulés par un LPS, mais sans venir en contact avec la substance test, et la teneur en macrophages ayant de l'ASC agrégé dans les macrophages dérivés de cellules iPS ayant un gène de type sauvage NLRP3 mesurée à l'étape (2) est équivalente à la teneur en macrophages ayant de l'ASC agrégé dans les macrophages dérivés de cellules iPS ayant un gène de type sauvage NLRP3 qui sont stimulés par un LPS, mais sans venir en contact avec la substance test.

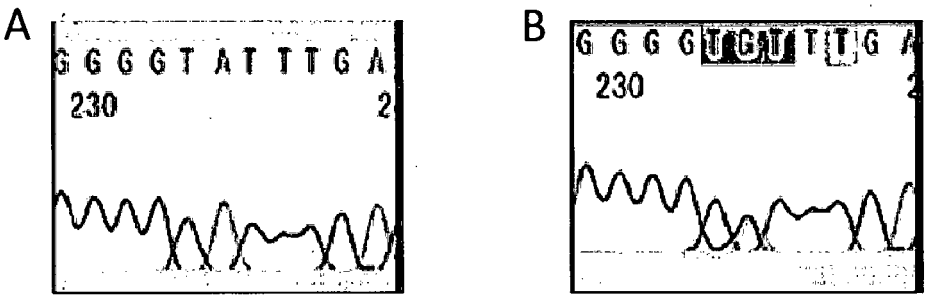
7. Procédé selon la revendication 6, dans lequel ledit médicament pour supprimer l'activité de l'inflammasome est un agent thérapeutique pour l'amiantose, la maladie d'Alzheimer, le diabète de type 2, les maladies cardiovasculaires athéroscléreuses, la goutte ou un syndrome périodique associé à la cryopyrine.

8. Procédé selon la revendication 6, dans lequel ledit gène mutant NLRP3 est un gène NLRP3 dans lequel l'adénine en position 1709 est mutée en guanine.

9. Procédé selon la revendication 6 (B) ou 6 (C), dans lequel des macrophages dérivés des cellules iPS ayant un gène de type sauvage NLRP3 sont en outre stimulés par de l'ATP.

10. Procédé selon la revendication 6 (B) ou 6 (C), dans lequel lesdites cellules iPS ayant un gène mutant NLRP3 et lesdites cellules iPS ayant un gène de type sauvage NLRP3 sont des cellules iPS dérivées de la même personne.

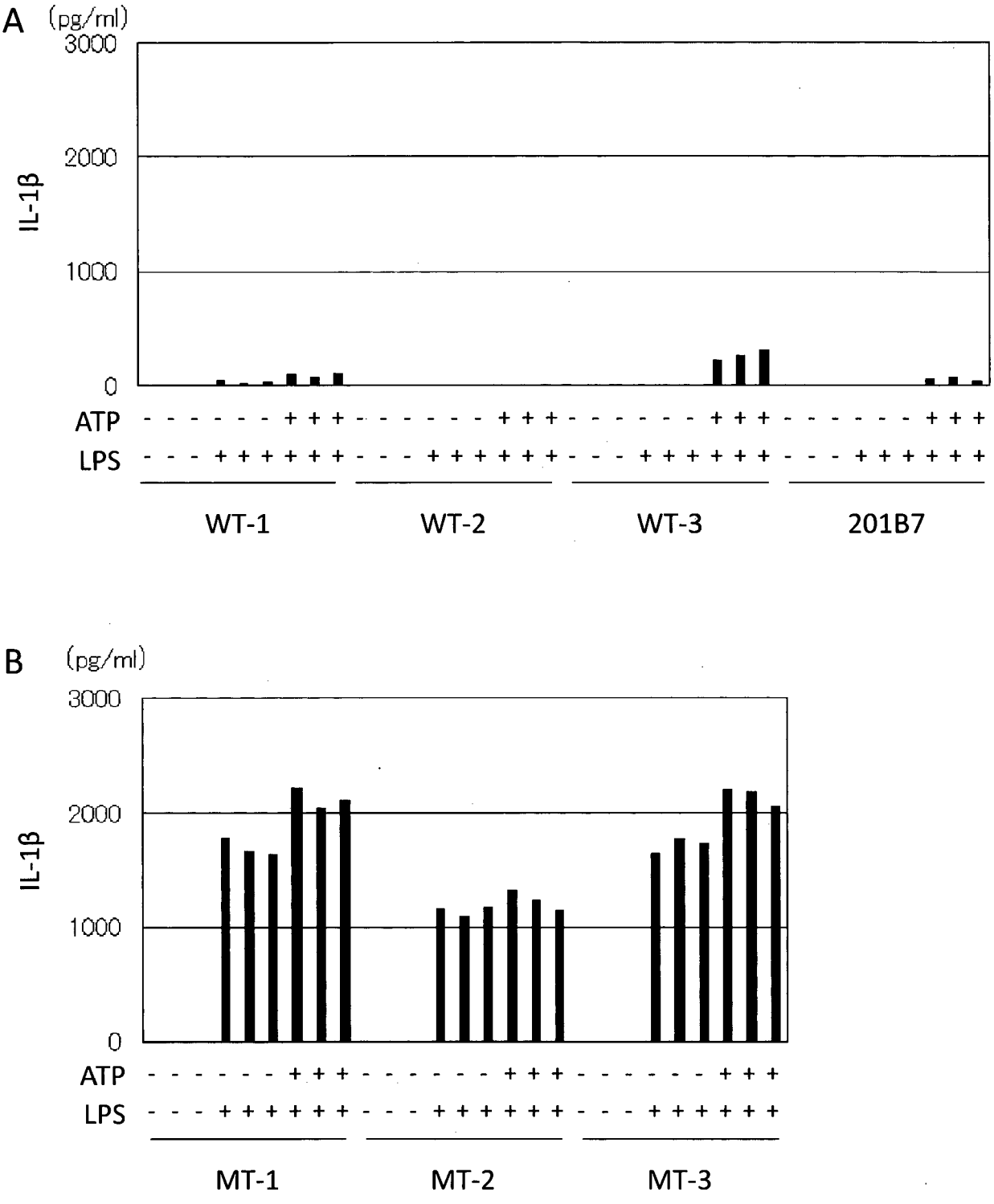
Fig. 1



Wild type

Mutant type

Fig. 2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2000
1000
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IL-1β
ATP
LPS
WT-1
WT-2
WT-3
201B7
B
(pg/ml)
3000
2000
1000
0
IL-1β
ATP
LPS
MT-1
MT-2
MT-3

Fig. 3

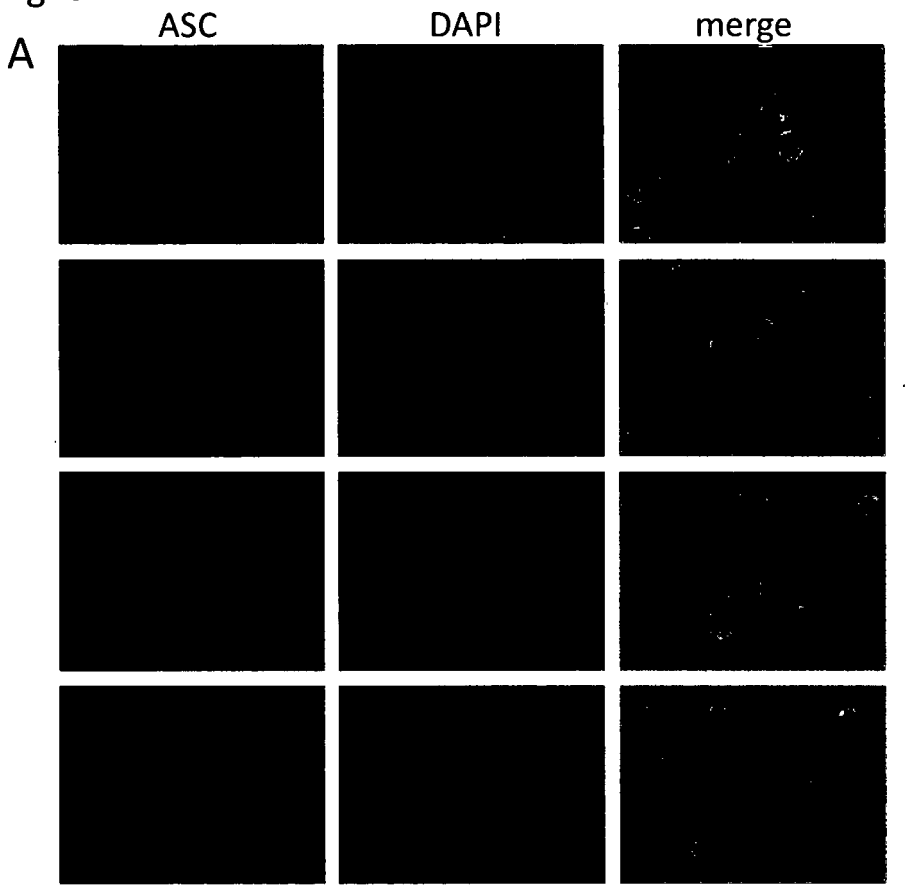
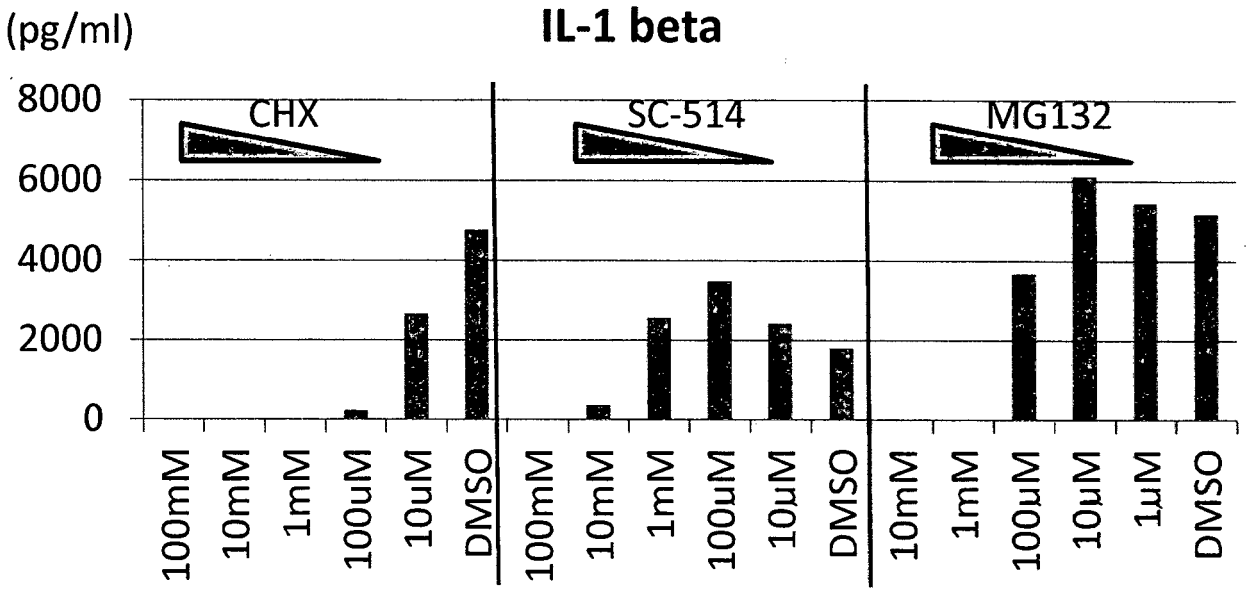

A
ASC
DAPI
merge
Wild type: no stimulation
B
ASC
DAPI
merge
Mutant type: stimulation with LPS and ATP

Fig. 4


(pg/ml)
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4000
2000
0
CHX
SC-514
MG132
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10mM
1mM
100uM
10uM
DMSO
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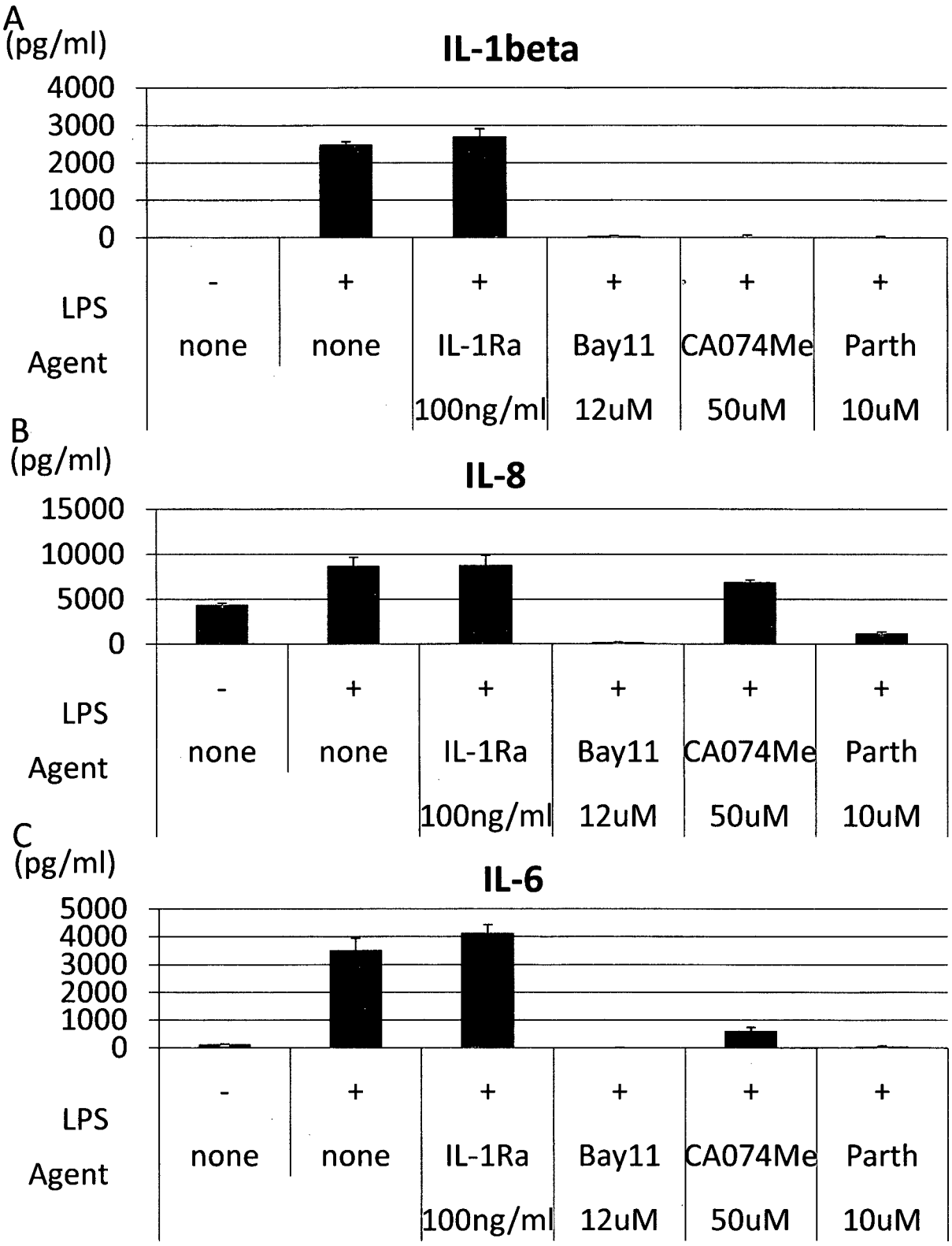
10mM
1mM
100uM
10uM
1uM
DMSO

Fig. 5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(pg/ml)
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+
+
+
+
+
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100ng/ml
Bay11
12uM
CA074Me
50uM
Parth
10uM
B
(pg/ml)
IL-8
15000
10000
5000
0
C
(pg/ml)
IL-6
5000
4000
3000
2000
1000
0

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